REMARKS

Reconsideration of this application is requested in view of the amendments to the specification, the amendments to the claims and the remarks presented herein.

The claims in the application are claims 23, 28 to 31, 35 to 39, 41 and 43, all other claims having been cancelled.

The specification has been amended to refer to Figs. 6A to 6C and indicate that the trademarks refer to silica based reversed-phase columns which are well known in the art as can be seen from the material filed herewith. With respect to the sequence numbering, it has been taken care of by the amendment of August 27, 2003 but if the Examiner deems otherwise, she is requested to be more specific. When addressing specifically the sequences disclosed in figure 5, please be informed that the upper sequence "RTTTAYRPPNAPILK" consists of SEQ ID No. 9 of the sequence listing and the sequence "ILKEPVHGV" consists of the sequence SEQ ID No. 114 of the sequence listing as filed.

Claims 23, 25, 26, 30, 36-38 and 43 stand rejected under 35 U.S.C. 112, first paragraph as being non-enabled by the specification for the lipopeptides and the epitopes

Applicants respectfully traverse this ground of rejection since it is deemed that the amended claims are completely enabled by the disclosure. First, it should be stated again

that, as it can be clearly understood from the specification, and more particularly the examples, the most efficient lipopeptides are those that comprise only one single lipid moiety, as it is illustrated in the examples by the lipopeptides comprising a single palmitoyl chain, also termed "mono-palm".

The synthesis of the mono-palmitoyl lipopeptides is disclosed in example 2 and Figure 4 shows that the mono-palmitoyl lipopeptide is more efficient than dipalmytoyl lipopeptide as regards the respective properties of inducing $CD8^+$ (see example 3, section 4, pages 16 and 17 of the specification). Further, the mono-palmitoyl derivatives are water-soluble, whereas the dipalmytoyl derivatives are soluble only in a water-DMSO mixture (see example 5, page 19 of the specification). Still further, it appears clearly in figure 6b, that the mono-palmitoyl lipopeptides are farther efficient than the dipalmytoyl lipopeptides in inducing a CTL response (see example 5, page 23). Yet further, figure 7a) shows that the better properties of the mono-palmitoyl lipopeptides than the dipalmytoyl lipopeptides to induce a $CD8^+$ response (see example 5, page 24, lines 10-15 of the specification). Also, figures 8A and 8B show the high potency of a mono-palmitoyl lipopeptide to induce the production of a $CD8^+$ response (see example 5, Section 3, pages 24 – 25 of the specification).

Further, Applicants are submitting a copy of an article from LEVY et al. (2205, Aids, vol. 19(3): 279-286) which further underlines the *in vivo* efficiency of the claimed lipopeptides for therapeutic immunisation of HIV-1 infected patients. As clearly stated in the right column of the Section "Discussion" of this article, this further study wherein the

claimed lipopeptide were administered to HIV-1 infected patients having stopped the chemical anti-viral treatment, *"provides proof of the concept that therapeutic immunisation before anti-viral cessation may contribute to the containment of HIV replication"*.

Therefore, withdrawal of this ground of rejection is requested.

Claims 23, 25, 26, 30, 35, 38 and 43 were rejected under 35 U.S.C. 112, second paragraph as being indefinite.

Applicants respectfully traverse these grounds of rejection as the amended claims are definite. Claim 38 has been amended as suggested by the Examiner and claim 26 has been incorporated into claim 23. Claim 25 has been cancelled and claim 23 has been amended to recite amino acid residues. Therefore, withdrawal of these grounds of rejection is requested.

Claims 23, 25, 26, 30, 35-38 and 43 were rejected under 35 U.S.C. 103 as being obvious over WO 95/19783 taken in view of 6 other references. The Examiner states that WO 95/19783 teaches covalent attachment of palmitic acid to peptides to increase immunogenicity, that attachment of Th epitopes will also increase immunogenicity, that an amino acid sequence containing a Th epitope can be linked to the CTL epitope, that the Th epitope may be linked at the N-terminus by a peptide spacer and that amino acid residues may be introduced at the N-terminus or C-terminus of a protein.

All the claims were further rejected under 35 U.S.C. 103 as being obvious over WO 95/19783 taken in view of 5 references as well as 6 other references.

Applicants respectfully traverse these obviousness rejections which were summarized as follows: that the one skilled in the art would have arrived at the claimed lipopeptides by combining, respectively (i) prior art documents disclosing lipopeptides that induce a CTL response, including against HIV antigens, (ii) prior art documents related to the induction of a CTL response against HIV and comprising a single lipid chain, and (iii) prior art documents related to protein constructs comprising spacer chain.

(i) The first class of documents encompass PCT Application No. WO 95/19783, the US Patent No. 5,993,823, DEPREZ et al. (1996), European Patent Application No. EP EP 346 022, RAMMENSEE et al. (1995), and BERZOFSKY et al. (1991).

(ii) The second class of prior documents include BEN MOHAMED et al.(1997), US Patent No. 6,015,564 (BOUTILLON et al.), and US Patent No. 5,871,746 (BOUTILLON et al.).

Applicants traverse these rejections for the reason that none of the prior art documents of the third class above disclose nor suggest to one skilled in the art any

spacer chain that is included in a presently claimed lipopeptide. US Patent No. 5,935,824 as well as US Application No. 2003/0162719 do not disclose any lipopeptide.

The US Patent No. 5,935,824 discloses a system for expressing fusion proteins, wherein the fusion protein can contain a hydrophilic spacer chain. Also, the US Application No. 2003/01 62719 discloses peptides that are coupled to aminoacid chains for transport a cross biological membranes. However, none of these prior art documents could induce the one skilled in the art to apply the hydrophilic spacer chains that they disclose to a lipopeptide construct.

Moreover, even if, in a purely artificial reasoning, the one skilled in the art would have applied one hydrophilic spacer chain disclosed in one of these prior art documents to a lipopeptide construct, he would still have not arrived to the claimed lipopeptides, because none of these two prior art documents disclose nor suggest to the one skilled in the art a spacer chain selected from the group consisting of GLY ARG and ARG GLY ARG. Therefore, withdrawal of these rejections is requested.

With respect to the obviousness double patenting rejection with respect to US Patent No. 5,871,746 or Patent No. 6,015,564, Applicants are filing a Terminal Disclaimer with respect to these patents which obviates this rejection.

In view of the amendments to the specification, amendments to the claims and the above remarks, it is believed that the claims point out Applicants'patentable contribution. Therefore, favorable reconsideration of the application is requested.

Respectfully submitted
Hedman and Costigan

Charles A. Muserlian #19,683
Attorney for Applicants
Tel. 212 302 8989

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Enclosures

Immunological and virological efficacy of a therapeutic immunization combined with interleukin-2 in chronically HIV-1 infected patients

Yves Lévy[a], Hanne Gahéry-Ségard[b], Christine Durier[c], Anne-Sophie Lascaux[a], Cécile Goujard[d], Vincent Meiffrédy[c], Christine Rouzioux[e], Raphaëlle El Habib[f], Maria Beumont-Mauviel[g], Jean-Gérard Guillet[b], Jean-François Delfraissy[d], Jean-Pierre Aboulker[c] and the ANRS 093 Study Group*

Objective: Several lines of evidence suggest that the immune system may control HIV-1 replication, but that it could fail in the long term. Strategies aimed to elicit specific immune responses may enable patients to contain virus replication.

Methods: HIV-1-infected patients were randomized to continue either their antiviral therapy alone (controls; $n = 37$) or with four boosts of vaccination combining ALVAC-HIV (vCP1433) and Lipo-6T vaccines (weeks 0, 4, 8, 12) followed by three cycles of subcutaneous interleukin-2 (weeks 16, 24, 32) (Vac-IL-2 group; $n = 34$).

Results: Of the Vac-IL-2 group, 15/32 (47%) exhibited a stable HIV p24 antigen-proliferative response compared with 8/33 (24%) controls ($P = 0.049$). After vaccination, 19/33 (58%) of the Vac-IL-2 group exhibited a multiepitopic HIV-1-specific CD4 cell proliferative response compared with 9/36 (25%) of controls ($P = 0.006$). The breadth and the magnitude of HIV-specific interferon-γ-producing CD8 T cells improved in the Vac-IL-2 group. After stopping antiviral drugs, 24% of the Vac-IL-2 group lowered their viral set point compared with 5% of controls ($P = 0.027$). Logistic-regression analysis demonstrated that vaccine-elicited immunological responses were predictive of virological control ($P = 0.046$ and 0.014 for stable and multiepitopic CD4 T cell responses, respectively).

Conclusion: This study provides proof of the concept that therapeutic immunization before antiviral drug cessation may contribute to the containment of HIV replication.

AIDS 2005, **19**:279–286

Keywords: vaccine-ALVAC, HIV, lipopeptide, immunotherapy, interleukin-2

Introduction

The rationale of therapeutic immunization in HIV infection is based on several lines of evidence suggesting that the immune system contributes to the long-term control of HIV-1 replication [1–4]. For example, in a subgroup of patients, called long-term non-progressors, HIV-specific detectable immune responses seem to be

From the [a]Hôpital Henri Mondor, Créteil, [b]INSERM Institut Cochin, Paris, [c]INSERM Service Commun 10, Villejuif, [d]Hôpital de Bicêtre, Le Kremlin-Bicêtre, [e]Unité de Virologie, Hôpital Necker, Paris, [f]Aventis Pasteur, Marcy l'Etoile, [g]Chiron, Suresnes and ANRS, Paris, France.

Correspondence to Pr Y. Lévy, Unité d'Immunologie Clinique, Hôpital Henri Mondor, 51 avenue du Maréchal de Lattre de Tassigny, 94010 Creteil Cedex, France.

E-mail: yves.levy@hmn.ap-hop-paris.fr

*See the Appendix for members of the ANRS 093 Study Group.

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associated with control of HIV replication to exceptionally low levels [3,4]. However, continued viral replication leading to a progressive immune destruction is the rule in a majority of patients. In the long term, although HIV-1-specific CD4 and CD8 cells may be detectable in patients at different time points of the disease, these cells are functionally impaired and fail to control viral replication after treatment discontinuation [5–9].

Whether a therapeutic immunization might help to induce specific cellular immune responses in HIV-infected patients remains debatable. To date, only limited studies have evaluated different therapeutic vaccines, such as inactivated Remune vaccine [10] or canarypox virus vaccine alone or combined with recombinant gp160 in chronically or newly HIV-infected patients [11–13]. In these studies, immunological responses to vaccination were transient and the dynamics of viral rebound after antiviral discontinuation in patients did not differ from historical controls [14].

In this study, we evaluated the immunogenicity of a therapeutic immunization strategy combining two different vaccines, recombinant ALVAC–HIV (vCP1433) and Lipo-6T (HIV-1 lipopeptides), followed by the administration of subcutaneous interleukin-2 (IL-2), in chronic HIV-infected subjects while treated with potent antiviral therapy. Although not competent to replicate in mammalian cells, ALVAC-HIV allows the presentation of peptides encoded by several HIV genes (*env*, *gag*, *pol* and *nef*) by antigen-presenting cells. Lipo-6T consists of five long amino acid sequences from Nef, Gag, Pol and one promiscuous epitope from tetanus toxoid used to stimulate CD4 T cell help. These peptides were modified in the C-terminal region by adding a lipid tail, which was shown to be useful for increasing the presentation of antigens by the immune system [15]. We show here that the therapeutic immunization strategy leads to HIV-1-specific T cell responses in chronically HIV-infected patients. In addition to *in vitro* immunological tests, the study evaluated whether this immune intervention altered the dynamics of viral replication in patients.

Methods

Patients, vaccines and study design

HIV-1-infected patients treated with combined antiviral regimens for at least 1 year were eligible. Patients who received subcutaneous IL-2 in a previous study [16] were also eligible provided the last cycle was administered at least 3 months prior study entry. The study was approved by the institutional ethics review boards and all patients gave written informed consent. Of 86 patients screened between June 2000 and April 2001, 71 patients were eligible and randomized. Therapeutic immunization combined $10^{6.6}$ median infectious dose (ID_{50}) of ALVAC-HIV and 3 mg HIV lipopeptides Lipo6-T (Aventis Pasteur, Lyon, France). ALVAC-HIV expresses several HIV genes: for gp120 (MN strain) and a part of the anchoring transmembrane region of gp41 (LAI strain); for the p55 polyprotein, expressed by *gag* (LAI strain); for a portion of *pol* encoding the protease; and for genes expressing cytotoxic T lymphocyte peptides from *pol* and *nef*. The Lipo-6T vaccine is a mixture of the tetanus toxoid TT-830–843 class II-restricted universal CD4 epitope combined with five peptides: Gag 17–35, Gag 235–284, Nef 66–97, Nef 116–145 and Pol 325–355 from HIV-1_{LAI}. Vaccines were given intramuscularly. Three subcutaneously IL-2 cycles (4.5×10^6 IU twice daily for 5 days) were given following immunization. Patients from both arms stopped antiviral drugs at week 40. Viral loads and CD4 T cell counts were monitored weekly for 4 weeks and bimonthly thereafter until the end of the trial at week 52. Subjects whose viral load was >50 000 copies/ml at week 44 or 46, or >10 000 copies/ml after week 48, were in virological failure and recommended to resume antiviral drugs. HIV-1 DNA levels in peripheral blood mononuclear cells (PBMC) were determined in a central laboratory using a modified version of the Amplicor Monitor test (Roche, Molecular Systems, Branchburg, New Jersey, USA) as previously described [17].

Laboratory methods

Immunological tests were performed for all patients at weeks 0, 16 and 36. Treatment group assignment was masked to immunological investigators. HIV-specific CD4 T cell lymphoproliferation response was assessed on fresh PBMC as previously described [18]. Antigens included 1 μg/ml of one of 11 HIV-1 long peptides (Neosystem, Strasbourg, France), 5 μg/ml Gag p24 protein (produced by *Escherichia coli*; Aventis Pasteur) and control antigens. The HIV-1 long peptides used were derived from Nef, Nef 66–97 (N1), Nef 117–145 (N2), Nef 182–205 (N3); from Gag, Gag 183–214 (G1), Gag 253–284 (G2), Gag 17–35 (G3); from Pol, Pol 325–353 (P1), Pol 325–342 (P2), Pol 335–355 (P3); and from Env, Env 303–335 (Env), Env MN 303–335 (EMN); their sequences can be found in a recent publication [19]. Results are reported as either counts per minute (cpm) or stimulation index (median [^{3}H]-thymidine uptake of cells stimulated with antigen/median incorporation of cells cultured in medium alone). A stimulation index ≥3 and ≥3000 cpm incorporated was considered as positive response. Evaluation of HIV-specific CD8 T cells producing interferon-γ (IFN-γ) was assessed on frozen cells and using an ELISPOT assay performed as described [18]. Antigens included 18 pools of 15-mer peptides covering HIV-1 Gag (11 pools), reverse transcriptase (four pools) and Nef (three pools) (Neosystem) at 2 μg/ml. The IFN-γ-producing cells were counted using an automated microscope (Zeiss, Le Pecq, France) and expressed as spot-forming cells (SFC). Numbers of SFC were normalized to 10^6 PBMC/well and averaged over triplicate wells. The number of specific SFC/10^6 PBMC

was calculated by subtracting the negative control value from the established SFC count. Positive responses were defined as greater than 100 SFC/10^6 PBMC over background and at least twofold the background value. The breadth of the response was the number of recognized pools among 18 pools tested and the magnitude was defined as the sum of positive responses to individual pools (total SFC/10^6 PBMC).

Statistical analysis

Primary efficacy endpoints were the proportion of patients responders to both HIV p24 antigen and at least one of 11 peptides from *gag, pol, env and nef* (see above). A sample size of 35 per group was calculated to provide 80% power, with a one-sided test and a type I error of 5% for detecting a 30% increase in the proportion of responders in the vaccinated group compared with control. Secondary endpoints were the evaluation of HIV-specific CD8 cell responses by IFN-γ ELISPOT assay and the proportion of patients in virological success (stopped antiviral drugs at week 40 and remained off therapy with plasma HIV RNA values <50 000 copies/ml at week 44 and <10 000 copies/ml at week 48 and thereafter until week 52). Parameters of the kinetics of plasma HIV RNA rebounds following antiviral drug discontinuation were analysed using the Wilcoxon rank-sum test for continuous data and the log-rank test for time-to-event data. For ELISPOT data, the proportion of responders were compared between control and vaccinated groups with Fisher and Wilcoxon one-sided tests.

Randomization was centralized and stratified by prior IL-2 status. CD4 cell proliferative responses and ELISPOT data were analysed on evaluable patients at each time point, which was an *a priori* decision and not related to treatment received. Analyses of immunological and virological data were performed on an intention-to-treat approach (with restrictions as above) by using the one-sided Fisher's exact test for proportions and the one-sided log-rank test for the time to failure variable (time was set up to 0 for non-interrupters). Relationship between proliferative responses and virological outcome was performed with two-sided Fisher exact test and logistic regressions, explaining virological success by treatment group alone, proliferative responses alone and both terms in the model, with an approach similar to that used in validation of surrogate markers. Nominal *P* values are presented and *P* values for proliferative responses to HIV-1 long peptides were adjusted for multiplicity (permutation resampling method). The software used for statistical analysis was SAS 8.0 (SAS Institute, Cary, North Carolina, USA).

Results

Safety and tolerability of the therapeutic immunization

A group of 71 patients with asymptomatic HIV-1 infection and CD4 T cell counts >350 × 10^6 cells/l and plasma HIV RNA <50 copies/ml and who had been previously treated with combined antiviral regimens alone or combined with subcutaneous IL-2 in a previous study [16] were eligible for this study. Patients were randomized to continue either their antiviral therapy alone (control group; $n = 37$) or combined with the administration of the two vaccines (Vac-IL-2 group; $n = 34$) at weeks 0, 4, 8 and 12. Following vaccinations, patients were administered three cycles of subcutaneous IL-2 (4.5 × 10^6 IU, twice daily for 5 days) at weeks 16, 24 and 32. One patient withdrew from the trial prior to receiving study treatment and was excluded from the analysis. Baseline characteristics were similar in the two groups (Table 1). All patients completed the vaccination schedule. All patients of this group received IL-2 cycles except two patients, who missed one IL-2 cycle. No patient was lost to follow-up at week 52. The vaccination strategy was safe and well tolerated. All local or systemic adverse events related to the immunization were mild or moderate and of short duration. Local or systemic adverse events related to IL-2 cycles were similar to those previously reported [16]. There was no deleterious effect of the immunization strategy on plasma viral load (Table 2).

Induction of HIV-specific-CD4 T cell proliferative responses by immunization

The CD4 T cell responses were evaluated using lymphoproliferative assays at different time points in the study. At baseline, the percentage of responders to p24

Table 1. Baseline characteristics of patients.

	Control	Vac-IL-2
Number	37	33
Median CD4 cell count [×10^6 cells/l (range)]	766 (361–2508)	713 (233–2160)
Median nadir CD4 cell count [×10^6 cells/l (range)]	290 (2–510)	262 (63–598)
Median CD8 cell count [×10^6 cells/l (range)]	855 (346–2559)	953 (211–1441)
Median plasma HIV RNA <50 copies/ml [No. (%)]	35 (95)	32 (97)
Median antiviral therapy duration [weeks (range)]	218 (75–642)	184 (71–592)
Median HIV-1 DNA [$\log_{10}$ copies/10^6 PBMC (range)]	2.68 (<1.85–3.85)	2.71 (<1.85–3.38)
No. patients with prior IL-2 treatment	9	5
Median IL-2 cycles with prior entry (range)	10 (8–11)	10 (5–10)
Median last IL-2 cycle [weeks (range)]	45 (22–74)	69 (18–93)

IL-2, interleukin-2; Vac-IL-2, ALVAC-HIV (vCP1433) and Lipo-6T vaccines plus IL-2 PBMC, peripheral blood mononuclear cells.

Table 2. Virological parameters after stopping and before reinitiating antiviral therapy or week 52.

	Control	Vac-IL-2	*P* value[a]
All patients			
HIV RNA <50 copies/ml at week 36 [No./total No. (%)]	32/37 (86)	32/33 (97)	0.13
Stop HAART at week 40 [No./total No.]	31/32	32/32	
Virological success at week 52 [No./total No. (%)][b]	2/37 (5)	8/33 (24)	0.027
Time to virological failure [median days (IQR)]	29 (28–88)	42 (27–55)	0.009
Patients who stopped antiviral therapy at week 40			
Time to first detectable viraemia [median days (range)]	13 (7–21)	14 (11–21)	0.16
Peak HIV RNA [median $\log_{10}$ copies/ml (IQR)]	5.2 (4.9–5.5)	4.9 (4.5–5.4)	0.082
Time to peak HIV RNA [median days (IQR)]	34 (25–42)	41.5 (28–56)	0.024
Maximum slope of rebound (median $\log_{10}$ copies/ml per days (IQR)]	0.27 (0.21–0.32)	0.23 (0.18–0.29)	0.067
Delay for resuming antiviral therapy (median days (IQR)]	54 (42–70)	69.5 (42–91)	0.11
Time to undetectable HIV RNA (median days (IQR)]	119 (87–194)	112 (76–147)	0.14
Median CD4 cell count at week 40 ($\times 10^6$ cells/l (IQR)]	851 (581–1048)	961 (714–1356)	0.042
Median nadir CD4 cells during interruption ($\times 10^6$ cells/l (IQR)]	520 (325–782)	651 (517–857)	0.026

IQR, interquartile range; Vac-IL-2, ALVAC-HIV (vCP1433) and Lipo-6T vaccines plus interleukin-2.
[a]Fisher test, Wilcoxon rank-sum test, log-rank test for the time variables (one-sided tests).
[b]Patients were considered as in virological success if they stopped antiviral drugs at week 40 and remained off therapy and with plasma HIV RNA values <50 000 copies/ml at week 44 and <10 000 copies/ml at week 48 and thereafter until week 52.

Gag antigen was 47% (15/32) and 57% (21/37) in the Vac-IL-2 and control groups, respectively. At weeks 16 and 36, the percentage of responders to p24 remained comparable in the two groups. However, longitudinal analysis showed that a higher proportion of the Vac-IL-2 patients maintained proliferative responses [15/32 (47%)] to p24 at both evaluations at weeks 16 and 36 than in the control group [8/33 (24%)] ($P = 0.049$) (Fig. 1). This difference between arms remained significant after adjustment on baseline responses (Cochran–Mantell–Haenszel test $P = 0.039$). In order to evaluate the breadth of proliferative responses to HIV antigens, lymphoproliferation response assays were performed using 11 long HIV peptides corresponding to Nef (N1, N2, N3), Gag (G1, G2, G3), Pol (P1, P2, P3) and two from Env (Fig. 1). At baseline, the percentages of responders to at least 1 of 11 HIV-1 long peptides were 41% (13/32) and 47% (18/37) in the Vac-IL-2 and control groups, respectively. At week 16, these percentages were 58% (19/33) and 25% (9/36) in the Vac-IL-2 and control groups, respectively ($P = 0.006$) (Fig. 1). At week 16, 68% (13/19) of the Vac-IL-2 group acquired new T cell responses against HIV-1 long peptides compared with 26% (5/19) of controls ($P = 0.022$) (Fig. 2a). The most frequently recognized peptides were G2, P1 and P2, which induced proliferative responses in a higher proportion of Vac-IL-2 patients than controls ($P = 0.019$, 0.001 and 0.019, for the three peptides, respectively) (Fig. 2b).

The effect of immunization on interferon-γ-producing CD8 T cell responses

The IFN-γ-producing CD8 T cells were evaluated using an ELISPOT assay and 18 pools of 15-mer peptides overlapping Gag, reverse transcriptase and Nef HIV-1 sequences. At entry, 50% of patients were responders to at least 1/18 pools in each group (16/32 in Vac-IL-2; 17/34 in controls) (Fig. 3). The number of responders did not vary between groups at weeks 16 and 36. However, the number of HIV-1 peptides recognized per patient tended to increase in the Vac-IL-2 group compared with controls at week 36, where 23% (7/31) of the Vac-IL-2 group recognized three pools at least compared with 5% (2/37) of control patients ($P = 0.066$) (Fig. 3a). For responders, a significant increase of the magnitude of those responses was noted in the Vac-IL-2 patients compared with controls. At week 36, the median total increases from baseline were 365 SFC/10^6 PBMC [95% confidence interval (CI), 122–777) for the Vac-IL-2 group and 138 SFC/10^6 PBMC (95% CI, −125 to 283) for the controls ($P = 0.046$) (Fig. 3b).

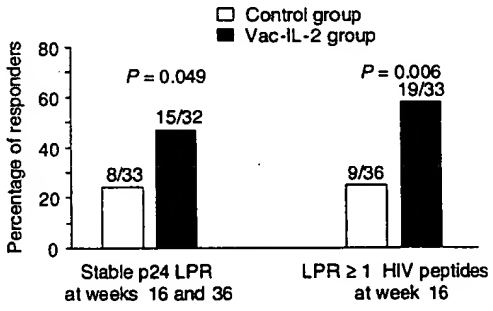

Fig. 1. Specific CD4 T cell lymphoproliferative responses to p24 Gag antigen and HIV-1 long peptides. The percentages and numbers of patients are shown above the columns for control (open columns) and Vac-IL-2 (dark columns) groups. The left set shows those with stable responses specific to HIV-1 p24 Gag antigen at both evaluations at weeks 16 and 36. The right set shows responders at week 16 to at least one of the 11 HIV-1 long peptides derived from Nef, Nef 66–97 (N1), Nef 117–145 (N2), Nef 182–205 (N3); from Gag, Gag 183–214 (G1), Gag 253–284 (G2), Gag 17–35 (G3); from Pol, Pol 325–353 (P1), Pol 325–342 (P2), Pol 335–355 (P3); and from Env, Env 303–335 (Env), Env MN 303–335 (EMN). Vac-IL-2, ALVAC-HIV (vCP1433) and Lipo-6T vaccines plus interleukin-2.

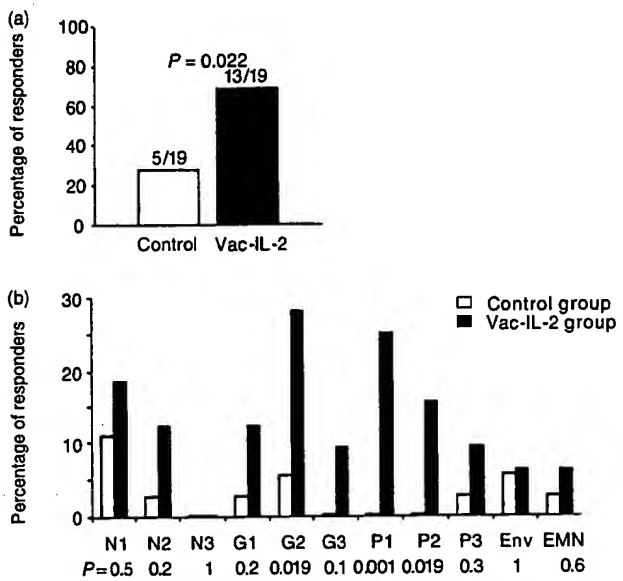

Fig. 2. Acquired specific CD4 T cell proliferative responses elicited by the vaccination. (a) Percentages and numbers of patients who developed new responses at week 16 against at least one HIV-1 long peptide among 11 tested (see sequences in Fig. 1). At baseline (week 0 prior immunization), 19 patients from each group were non-responders to those HIV-1 long peptides. (b) Comparison of the percentage of new responders at week 16 to each of the 11 long HIV-1 peptides (see sequences in Fig. 1) in control and Vac-IL-2 groups. Responses to G2, P1 and P2 peptides were significantly induced by the vaccination compared with controls. Vac-IL-2, ALVAC-HIV (vCP1433) and Lipo-6T vaccines plus interleukin-2.

The effect of immunization on HIV rebound after cessation of antiviral drugs

At week 40, 8 weeks after the end of the therapeutic immunization, it was proposed that patients who kept undetectable plasma viral load stopped their antiviral therapy: 97% (32/33) in the Vac-IL-2 group and 86% (32/37) in the control group ($P = 0.13$) were eligible for stopping antiviral drugs. Parameters of the kinetics of the viral rebound during the period of antiviral treatment interruption and after resuming antiviral therapy are shown in Table 2. At week 52, the proportion of patients in virological success, according to strict criteria defined at the beginning of the study (see Table 2), was significantly higher in the Vac-IL-2 group [8/33 (24%)] than in controls [2/37 (5%)] ($P = 0.027$) (Table 2). The time for resuming antiviral drugs, in patients who were in virological failure, was also significantly delayed in the Vac-IL-2 group compared with the control group (median of 42 and 29 days, respectively; $P = 0.009$) (Fig. 4).

Immunological responses to HIV antigens and virological control in vaccinated patients

Univariate and multivariate logistic regression analyses were performed for the immunological parameters associated with the virological control noted at week 52. For CD8 T cell responses, the results showed only a trend towards an association between the breadth of these responses at week 36 (at least three versus less than three positive pools) and the time to virological failure was noted ($P = 0.055$). For CD4 cell proliferative responses, univariate analyses showed that the odds ratio (OR) of virological success was higher in patients who were responders to at least one HIV peptide at week 16 than in non-responders (OR, 7.8; $P = 0.014$) and in patients with stable responses to HIV p24 antigen than in non-stable responders (OR, 4.6; $P = 0.046$). In the multivariate logistic regression analyses, the OR values comparing treatment groups were no longer significant when stable p24 antigen and multiepitopic (to at least one HIV peptide) responses were included in the model ($P = 0.16$ for both comparisons), demonstrating that the effect of the vaccine regimen on the virological success was partially correlated with the immunological responses elicited.

Discussion

The study reported here showed that a therapeutic immunization strategy combining ALVAC-HIV and Lipo-6T vaccines followed by the administration of IL-2 induced sustained and broad CD4 T lymphocyte immune responses to HIV antigens in chronically HIV-infected patients. Although less dramatically, IFN-γ-producing CD8 T cell responses to HIV peptides tended also to augment in these patients compared with controls. However, since the clinical relevance of *in vitro* immunological responses is still unknown, the impact of therapeutic immunization on the control of HIV replication after stopping antiviral drugs was evaluated. Using rigorous prospective criteria for the definition of virological control and for resuming antiviral treatment, 24% (8/33) of the Vac-IL-2 group compared with 5% (2/37) of controls ($P = 0.027$) remained off antiviral drugs 3 months after stopping these drugs. Moreover, the vaccine strategy significantly improved several parameters of the kinetics of HIV RNA rebound occurring during the period off antiviral therapy (Table 2). Although the final endpoint of this study was planned at week 52, 3 months after stopping antiviral drugs, all patients were followed 1 year beyond the study endpoint. This long-term follow-up showed that this effect was sustained since the overall median duration of the period off treatment extended to 35 weeks. This study has provided proof of the concept that an immunogenic vaccination strategy may result in a decrease of the viral set point following antiviral drug withdrawal in chronically HIV-infected patients.

In this study, we were interested to combine two different vaccines in order to improve the immunogenicity of the immunization strategy and to extend the breadth of

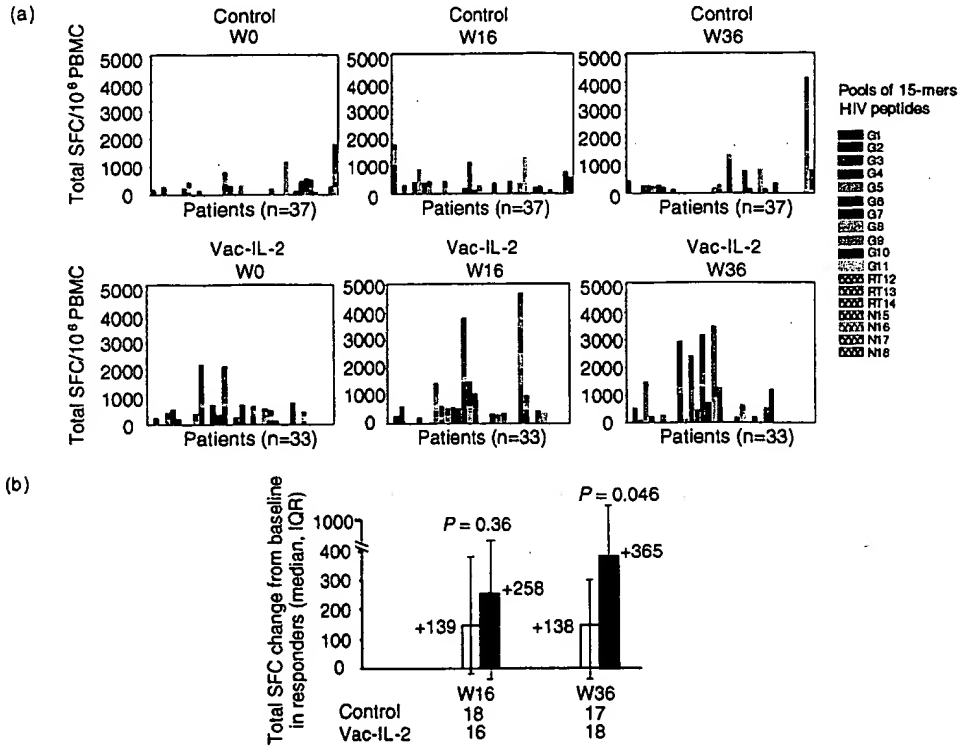

Fig. 3. HIV-1 specific CD8 T cell responses as measured with interferon-γ ELISPOT assay. (a) For each subject, the magnitude of the response as assessed by the total number of SFC/10^6 PBMC against each HIV peptide pools at each time point: the total of positive responses to HIV Gag (11 pools of 15-mers peptides), reverse transcriptase (four pools) and Nef (three pools). (b) For patients responders to at least one HIV pool, evolution of the changes from baseline of the magnitude of the responses at week 16 (after vaccination) and at week 36 (after IL-2 administration). W0, week zero etc.; Vac-IL-2, ALVAC-HIV (vCP1433) and Lipo-6T vaccines plus interleukin-2; SFC, spot-forming cells; PBMC, peripheral blood mononuclear cells.

vaccine-induced immunity. This was supported by the fact that the two vaccines used in this study shared several HIV sequences and by recent data showing that lipopeptide vaccines contained CD8 and CD4 T and B cell epitopes, inducing antibody, CD4 T cell and CD8 responses in seronegative volunteers [18–20]. The rationale for the combination of vaccines and IL-2 is supported by several clinical and experimental considerations. Although the clear effect of IL-2 on the restoration of immune function is still under discussion [21], several studies have shown that intermittent IL-2 therapy may help to restore quantitatively and qualitatively T cell functions [16,22,23]. Recent studies have shown that IL-2 therapy may help to increase perforin and granzyme expressions in CD8 T cells purified from HIV-infected patients [24]. Of note, although HIV-specific IFN-γ-producing T cell responses increased weakly after immunization, we found here that the magnitude of HIV-specific effector CD8 responses became significantly higher after IL-2 administration in the Vac-IL-2 group compared with the controls. Finally, since the beginning of our study, several experimental models have shown that administration of IL-2 combined with DNA or recombinant viral vaccines in macaque models of SIV infection lead to augmented T cell-specific immune responses [25,26]. This positive effect of IL-2 was associated with a better control of viraemia either after infectious viral challenge or after antiviral drug withdrawal in preventive or therapeutic vaccination [25,26]. These results in experimental models, as well as the results reported here, reinforced the potential interest of this cytokine in the setting of therapeutic immunization.

Data from this randomized study showed that the combination of immunization and IL-2 contributed to the containment of virological replication once antiviral drugs were stopped. One limitation of our study is that the complexity of the therapeutic immunization strategy makes it difficult to ascribe the effects of each component in the control of viral load in patients after antiviral cessation. Instead, this study intended to examine the concept that an immune intervention may help to contain the viral set point in patients. However, the design of this study allowed us to determine the immunogenicity of the vaccination before and after the administration of IL-2 and also the impact of the combination on the viral load control. Regarding the interpretation of our results, we would like to emphasize the following points. First, as shown here for patients from the control group, and in a series of published studies, long-term suppression of viral

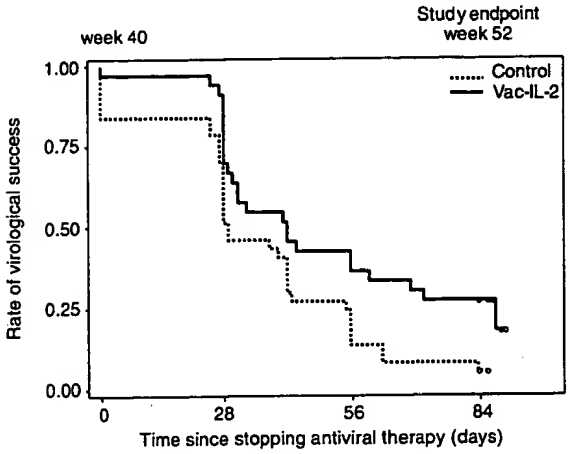

Fig. 4. Kaplan–Meier curves of time to virological failure. At week 52, 24% and 5% of patients were in virological success in the Vac-IL-2 and control groups, respectively ($P = 0.027$). The time to virological failure was significantly delayed in the Vac-IL-2 group compared with the control group ($P = 0.009$ in an intent-to-treat analysis; $P = 0.034$ when considering only patients who stopped antiviral therapy). Vac-IL-2, ALVAC-HIV (vCP1433) and Lipo-6T vaccines plus interleukin-2.

replication by potent antiviral therapy does not confer *per se* to patients the ability to contain virus replication once therapy is withdrawn. Second, several randomized phase II clinical studies have shown that IL-2 therapy combined with antiviral drugs has no impact on plasma viral load [16,22,23]. Moreover, despite the fact that some IL-2-treated patients were shown to have a low level of HIV burden in their CD4 T cell pools [27], the kinetics of HIV-1 rebound in these patients did not appear different from those who were treated with antiviral drugs alone [28]. Third, precise analysis of the immunological parameters elicited by the immunization showed that HIV-specific CD4 T cell responses elicited at week 16, before the administration of IL-2, were associated with the host control of virus rebound. This last result provides interesting indications of the immune correlates needed to be achieved for successful therapeutic immunization in chronically infected patients. Finally, further studies aimed to dissect the respective role of IL-2 and immunization in the control of virus replication are needed and would help to define more feasible and less-restricting schedules of vaccination.

The long-term clinical benefit of therapeutic immunization in HIV infection is still not demonstrated. Previous randomized phase I/II or III [29] studies failed to demonstrate any impact of the immunization strategy on the natural history of the disease. However, the majority of these studies had drawbacks, such as limited number of patients, no control group, or had been conducted in the setting of non-optimal antiviral therapy. Although there is as yet no consensus upon criteria for the clinical efficacy of therapeutic immunization, the capacity to stimulate HIV-specific immune responses and to control HIV replication after treatment interruption in chronically infected patients might be promising. This study provides proof of the concept that therapeutic immunization before antiviral cessation may contribute to the containment of HIV replication. We believe that these findings would help in the development of therapeutic immunization strategies in the treatment of HIV disease, and also in other chronic viral diseases.

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References

1. Borrow P, Lewicki H, Hahn BH, Shaw GM, Odstone MB. **Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection.** *J Virol* 1994; **68**:6103–6110.
2. Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, *et al.* **Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome.** *J Virol* 1994; **68**:4650–4655.
3. Rosenberg ES, Billingley JM, Caliendo AM, Boswell SL, Sax PE, Kalams SA, Walker BD. **Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia.** *Science* 1997; **278**:1447–1450.
4. Cao Y, Qin L, Zhang L, Safrit J, Ho DD. **Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection.** *N Engl J Med* 1995; **332**:201–208.
5. Lieberman J, Shankar P, Manjunath N, Andersson J. **Dressed to kill? A review of why antiviral CD8 T lymphocytes fail to prevent progressive immunodeficiency in HIV-1 infection.** *Blood* 2001; **98**:1667–1677.
6. Appay V, Nixon DF, Donahoe SM, Gillespie GM, Dong T, King A, *et al.* **HIV-specific CD8+ T cells produce antiviral cytokines but are impaired in cytolytic function.** *J Exp Med* 2000; **192**:63–75.
7. Goepfert PA, Bansal A, Edwards BH, Ritter GD Jr, Tellez I, McPherson SA, *et al.* **A significant number of human immunodeficiency virus epitope-specific cytotoxic T lymphocytes detected by tetramer binding do not produce gamma interferon.** *J Virol* 2000; **74**:10249–10255.
8. Champagne P, Ogg GS, King AS, Knabenhans C, Ellefsen K, Nobile M, *et al.* **Skewed maturation of memory HIV-specific CD8 T lymphocytes.** *Nature* 2001; **410**:106–111.
9. Carcelain G, Tubiana R, Samri A, Calvez V, Delaugerre C, Agut H, *et al.* **Transient mobilization of human immunodeficiency virus (HIV)-specific CD4 T helper cells fails to control virus rebounds during intermittent antiretroviral therapy in chronic HIV type 1 infection.** *J Virol* 2001; **75**:234–241.
10. Robbins GK, Addo MM, Troung H, Rathod A, Habeeb K, Davis B, *et al.* **Augmentation of HIV-1-specific T helper cell responses in chronic HIV-1 infection by therapeutic immunization.** *AIDS* 2003; **17**:1121–1126.
11. Tubiana R, Gomard E, Fleury H, Gougeon ML, Mouthon B, Picolet H, *et al.* **Vaccine therapy in early HIV-1 infection using a recombinant canarypox virus expressing gp160MN (ALVAC-HIV): a double-blind controlled randomized study of safety and immunogenicity.** *AIDS* 1997; **11**:819–820.
12. Goh LE, Perrin L, Hoen B, Cooper D, Phillips A, Janossy G, for the QUEST Study Group. **Study protocol for the evaluation of the potential for durable viral suppression after quadruple HAART with or without HIV vaccination: The Quest study.** *HIV Clin Trials* 2001; **186**:634–643.

13. Markowitz M, Jin X, Hurley A, Simon V, Ramratnam B, Louie M, Deschenes GR, *et al.* **Discontinuation of antiretroviral therapy commenced early during the course of human immunodeficiency virus type 1 infection, with or without adjunctive vaccination.** *J Infect Dis* 2002; **186**:634–643.
14. Jin X, Gao X, Ramanathan M Jr, Deschenes GR, Nelson GW, O'Brien SJ. **Safety and immunogenicity of ALVAC vCP1452 and recombinant gp160 in newly human immunodeficiency virus type 1-infected patients treated with prolonged highly active antiretroviral therapy.** *J Virol* 2002; **76**:2206–2216.
15. Klinguer C, David D, Kouach M, Wieruszeski JM, Tartar A, Marzin D, *et al.* **Characterization of a multi-lipopeptides mixture used as an HIV-1 vaccine candidate.** *Vaccine* 1999; **18**:259–267.
16. Levy Y, Durier C, Krzysiek R, Rabian C, Capitant C, Lascaux AS, and the ANRS 079 Study Group. **Effects of interleukin-2 therapy combined with highly active antiretroviral therapy on immune restoration in HIV-1 infection: a randomized controlled trial.** *AIDS* 2003; **17**:343–351.
17. Burgard M, Izopet J, Dumon B, Tamalet C, Descamps D, Ruffault A, *et al.* **HIV RNA and HIV DNA in peripheral blood mononuclear cells are consistent markers for estimating viral load in patients undergoing long-term potent treatment.** *AIDS Res Hum Retroviruses* 2000; **16**:1939–1947.
18. Pialoux G, Gahery-Segard H, Sermet S, Poncelet H, Fournier S, Gerard L, *et al.* **Lipopeptides induce cell-mediated anti-HIV immune responses in seronegative volunteers.** *AIDS* 2001; **15**:1239–1249.
19. Gahery-Segard H, Pialoux G, Figueiredo S, Igéa C, Surenaud M, Gaston J, *et al.* **Long-term specific immune responses induced in humans by a human immunodeficiency virus type 1 lipopeptide vaccine: characterization of the CD8+ T-cell epitopes recognized.** *J Virol* 2003; **77**:11220–11231.
20. Gahery-Segard H, Pialoux G, Charmeteau B, Sermet S, Poncelet H, Raux M, *et al.* **Multiepitopic B- and T-cell responses induced in humans by a human immunodeficiency virus type 1 lipopeptide vaccine.** *J Virol* 2000; **74**:1694–1703.
21. Valdez H, Mitsuyasu R, Landay A, Sevin SA, Chan ES, Spritzler J, *et al.* **Interleukin-2 Increases CD4+ lymphocyte numbers but does not enhance responses to immunization: results of A5046s.** *J Infect Dis* 2003; **187**:320–325.
22. Kovacs JA, Vogel S, Albert JM, Falloon J Jr, Davey RT, Walker RE, *et al.* **Controlled trial of interleukin-2 infusions in patients infected with the human immunodeficiency virus.** *N Engl J Med* 1996; **335**:1350–1358.
23. Levy Y, Capitant C, Houhou S, Carriere I, Viard JP, Goujard C, *et al.* **Comparison of subcutaneous and intravenous interleukin-2 in asymptomatic HIV-1 infection: a randomised controlled trial.** *Lancet* 1999; **353**:1923–1929.
24. Zou W, Foussat A, Capitant C, Durand-Gasselin I, Bouchet L, Galanaud P, *et al.* **Acute activation of CD8+ T lymphocytes in interleukin-2-treated HIV-infected patients. ANRS-048 IL-2 Study Group.** *J Acquir Immune Defic Syndr* 1999; **22**:31–38.
25. Barouch DH, Santra S, Schmitz JE, Kuroda MJ, Fu TM, Wagner W, *et al.* **Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination.** *Science* 2000; **290**:486–492.
26. Tryniszewska E, Nacsa J, Lewis MG, Silvera P, Montefiori D, Venzon D, *et al.* **Vaccination of macaques with long-standing SIVmac251 infection lowers the viral set point after cessation of antiretroviral therapy.** *J Immunol* 2002; **169**:5347–5357.
27. Chun TW, Engel D, Mizell SB, Hallahan CW, Fischette M, Park S Jr *et al.* **Effect of interleukin-2 on the pool of latently infected, resting CD4+ T cells in HIV-1-infected patients receiving highly active anti-retroviral therapy.** *Nat Med* 1999; **5**:651–655.
28. Davey RT Jr, Bhat N, Yoder C, Chun TW, Metcalf JA, Dewar R, *et al.* **HIV-1 and T cell dynamics after interruption of highly active antiretroviral therapy (HAART) in patients with a history of sustained viral suppression.** *Proc Natl Acad Sci USA* 1999; **96**:15109–15114.
29. Kahn JO, Cherng DW, Mayer K, Murray H, Lagakos HS. **Evaluation of HIV-1 immunogen, an immunologic modifier, administered to patients infected with HIV having 300 to 549 $\times 10^6$/l CD4 cell counts: a randomized controlled trial.** *JAMA* 2000; **284**:2193–2202.

Appendix

Scientific Committee: Y. Lévy, C. Goujard, J. F. Delfraissy, P. M. Girard, B. Hoen, L. Weiss, C. Rouzioux, J. G. Guillet, H. Gahéry-Ségard, A. Venet, Y. Taoufik, H. Chavez, J. P. Aboulker, V. Meiffrédy, A. Uludag, C. Durier, F. Marcellin, R. El Habib, M. P. Richard, M. Beumont, M. J. Commoy.

Participating clinical departments: Hôpital Henri Mondor, Créteil (Y. Lévy, A. Sobel, A.-S. Lascaux, N. Brahimi, P. Lesprit, C. Jung); Hôpital de L'Archet, Nice (J.-P. Cassuto, C. Ceppi); Hôpital Bicêtre, Le Kremlin Bicêtre (J.-F. Delfraissy, C. Goujard, D. Quertainmont, C. Godin, A. Nguyen-Wartel, M.-T. Rannou); Hôpital de l'Hôtel-Dieu, Angers (J.-M. Chennebault, J. Loison, E. Vivien); CHU Côte de Nacre, Caen (C. Bazin, M. Six, R. Verdon, P. Goubin); Hôpital Saint-Louis, Paris (E. Oksenhendler, L. Gérard, M. Martinie); Hôpital Sainte-Marguerite, Marseille (J.-A. Gastaut, T.-T. Dinh; G. Fabre); Hôpital de la Conception, Marseille (H. Gallais, I. Ravaux, M.-J. Gallais); Hôpital Louis Mourier, Colombes (E. Mortier, M. Bloch, C. Chandemerle); CHU Hôtel-Dieu, Nantes (F. Raffi, B. Bonnet, M. Sicot); CHU Hôpital Saint-Jacques, Besançon (B. Hoen, G. Achard, F. Coquet); Hôpital Antoine Béclère, Clamart (F. Boué, C. Pignon, A.-M. Delavalle); Hôpital Hôtel-Dieu, Lyon (C. Trépo, L. Cotte, V. Thoirain, C. Brochier); CHU Brabois, Nancy (T. May, L. Boyer); Centre Hospitalier Emile Müller, Mulhouse (G. Beck-Wirth, M. Benomar).

Immunological group: Institut Cochin, INSERM U567, Paris (J.-G. Guillet, H. Gahéry-Ségard, B. Charmeteau, M. Surenaud).

Data and Safety Monitoring Board: D. Emilie, A. Laplanche, B. Masquelier, C. Michon.

Coordinating Trial Centre: INSERM SC10, Villejuif [J.-P. Aboulker, V. Meiffrédy, A. Uludag, M. Resch, C. Levy, C. Durier (statistics), S. Izard (data management)].

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SB-CN	80A	180 m²/g	80°C	1.0-8.0	No	4.0%
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Phenyl	5LK102	5LK105	5LK110	5LK112	5LK115	5LK120	5LK125
SAX	5LN102	5LN105	5LN110	5LN112	5LN115	5LN120	5LN125
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A Quarterly Technical Newsletter *Winter 2003*

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The last two issues of *Grace Vydac Advances* have emphasized proteomics and LC/MS applications of VYDAC columns. In this issue we present articles illustrating a variety of peptide and protein analytical and preparative applications of importance in biochemical science.

*www.uspto.gov, January 22, 2003

Reversed-Phase Separation of Stereomeric Peptides on VYDAC® 218TP104 Column

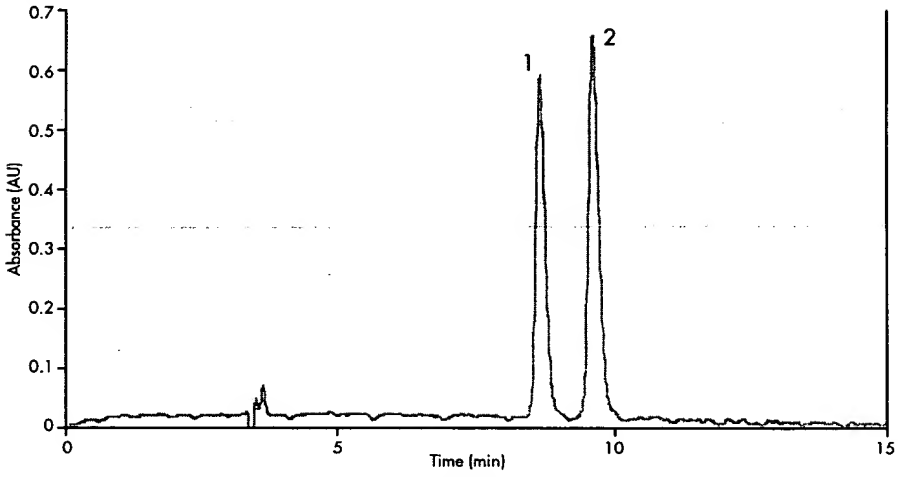

Figure 1. *HPLC separation of a mixture of H-Gly-L-Ser-Phe-NH_2 (1) and H-Gly-D-Ser-Phe-NH_2 (2). Column: VYDAC 218TP104 (C18, 10 µm, 300 Å, 4.6 mm ID x 250 mm). Mobile phase: A = 0.1% TFA/water. B = 0.1% TFA/acetonitrile. Gradient: linear from 5% to 15% B over 15 min. Flow: 1 mL/min. Detection: UV, 210 nm.*

HPLC Analysis of Stereomeric Peptides

In a recent poster, "Racemization Studies on Novel Cl-HOBt-based Coupling Reagents," presented at the 27th European Peptide Symposium in Sorrento, Italy, August 31 to September 6, 2002, Armida Di Fenza and Paolo Rovero of the University of Salerno reported using a VYDAC C18 reversed-phase column in their analyses. The ability of the VYDAC column to separate the stereomeric peptides H-Gly-L-Ser-Phe-NH_2 and H-Gly-D-Ser-Phe-NH_2 (Fig. 1) was key to its use in assessing the ability of novel coupling reagents to produce synthetic peptide bonds with minimal racemization of the resulting product.

Data courtesy of:

Armida Di Fenza and Paolo Rovero
Dept. of Pharmaceutical Sciences
University of Salerno
Via Ponte don Melillo 11C
I-84084 Fisciano, Salerno, Italy
Tel: [+39] 089 962809
Fax: [+39] 089 962828

VYDAC C4 Columns Aid in Chemical Synthesis of Model Protein

Also at the 27th European Peptide Symposium in Sorrento, Italy, August 31 to September 6, 2002, Neeraj Chopra, Duhee Bang, and Stephen B.H. Kent of the University of Chicago reported the complete chemical synthesis and folding of Crambin, a very hydrophobic 4716-dalton protein containing 46 amino acid residues with six Cys residues in three disulfide bonds. Crambin, which occurs naturally in seeds of the plant *Crambe abyssinica*, is of interest as a model for ultra-high-resolution (0.54 Å) X-ray crystallography, for developing advanced NMR methods for determining protein structure, and for evaluating theoretical approaches to calculation of protein folding. Producing Crambin in quantity using recombinant techniques is problematic because of its very hydrophobic nature.

Initially two component peptides – corresponding to Crambin amino acid residues 1-31 and 32-46 respectively – were produced by solid phase peptide synthesis. The peptide corresponding to residues 1-31 was synthesized as a thioester. Each of these peptides was purified by reversed-phase HPLC on a VYDAC C4 10 mm ID x 250 mm semi-prepreparative column (Cat.No. 214TP510) using conditions of 4 mL/min flow rate and 1%/min linear gradient of solvent B (0.08% TFA in ACN) in solvent A (0.1% TFA in water). Peptide purifications were verified (Figs. 2 and 3) by HPLC analysis on a VYDAC C4 4.6 mm ID x 250 mm analytical column (Cat.No. 214TP54) and electrospray MS analysis to confirm molecular weight. Conditions for analytical chromatography were 1 mL/min flow rate and a 2%/min linear gradient of the same mobile phase components as for purification.

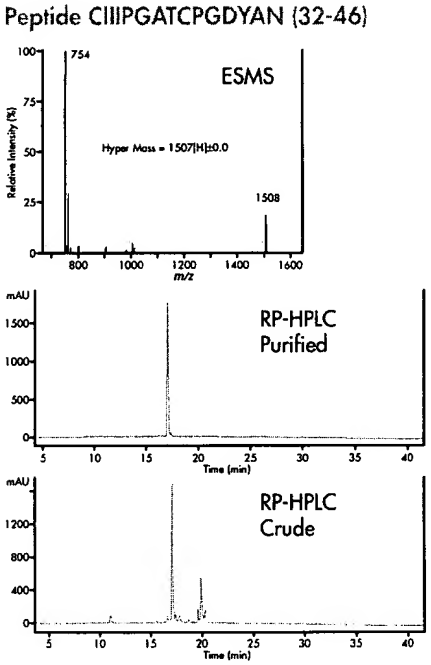

Figure 2. Reversed-phase and ESMS analysis of crude and purified peptide (32-36) on VYDAC 214TP54 C4 column.

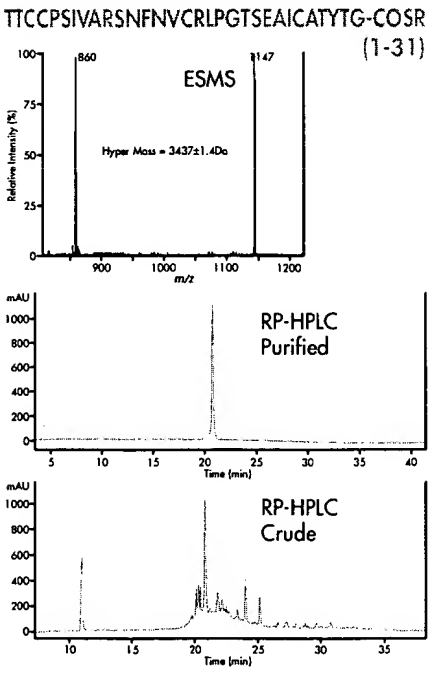

Figure 3. Reversed-phase and ESMS analysis of crude and purified peptide (1-31) thioester on VYDAC 214TP54 C4 column.

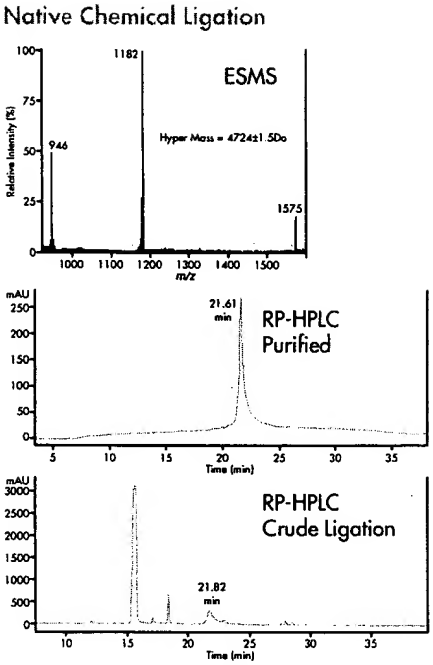

Figure 4. Reversed-phase and ESMS analysis of ligated synthetic Crambin (before folding) on VYDAC 214TP54 C4 column.

The two purified synthetic peptides were joined by thioester-mediated chemical ligation in solution to form the complete polypeptide chain of Crambin, which was also purified by HPLC on the VYDAC C4 semipreparative column (Fig. 4).

The polypeptide was then folded and disulfide bonds formed at pH 8 in aqueous buffer containing 2 M guanidine HCl, 8 mM cysteine and 1 mM cystine. The folded protein was again purified on the VYDAC C4 semi-preparative column and analyzed by HPLC and MS (Fig. 5). Note the increase in reversed-phase retention time with folding from 21.61 minutes to 22.38 minutes.

W. R. Grace, Silica Products, and Chromatography

In March 2001, The Separations Group, Inc., manufacturer of VYDAC HPLC columns and adsorbents, was acquired by W. R. Grace & Co.-Conn. and combined into the company's Silica Products business. Shortly thereafter, the Grace Vydac business unit was formed, combining Silica Products' existing chromatographic gel business with The Separations Group's column expertise.

W. R. Grace enjoyed a long and varied history leading up to its current status as a global supplier of specialty chemicals. The company began business in 1854 in Peru as an international shipping supply operation. It relocated to New York City in 1865. During the next century, the company grew into a diversified shipping, trading, and financial services company. Some highlights of that time include operating steamships between the Americas and Europe, establishing Grace National Bank, the forerunner of Marine Midland Bank, sending the first steamship through the Panama Canal in 1914, and initiating air travel to and from Latin America in 1928 as part of a joint venture with Pan American Airways. Company founder William Russell Grace was elected mayor of New York for noncontiguous terms in 1880 and 1884 and in that capacity was privileged to accept the Statue of Liberty as a gift from the people of France.

Davison Chemical, forerunner of today's Silica Products business, was originally founded as Davison, Kettlewell & Co. in 1832, in Baltimore, Maryland. Silica gel was patented in 1919 and originally used for adsorption of vapors and gases in gas-mask canisters during World War I. In the early 1920's Davison began selling silica gel and developed new applications such as air drying, refrigeration, and packaging desiccants. However, silica gel did not receive wide acclaim until World War II, when Davison developed the silica technology to meet three compelling wartime needs: first, as a dehydrating agent to protect military equipment from moisture damage; second, as a fluid cracking catalyst for producing high octane gasoline; and third, as a catalyst support, integral in the manufacture of synthetic rubber.

Davison was acquired by W. R. Grace in 1954. During the next 40 years, Grace acquired a variety of businesses before identifying and focusing on its core businesses of silica, catalysts, and construction materials. The Silica Products group has continued to expand its applications for silica and has become recognized for superior technical service, strong applications technology, and global customer support. With recent acquisitions, Silica Products now offers silica gels, colloidal and precipitated silicas, separations media and columns for chromatography, and molecular-sieve adsorbents that enhance manufacturing processes and end-products in a wide range of biotechnology, pharmaceutical, digital media, industrial, and consumer applications. The company has a strong global presence with research, technical service, manufacturing and sales offices strategically located around the world. Since the acquisition nearly two years ago, the company has been able to offer Grace Vydac customers:

- Improvements in manufacturing controls and documentation to assure reliably consistent and reproducible products.
- Improved availability of finished products for prompt delivery.
- Improved responsiveness to customer needs, exemplified by the new lines of VYDAC nano-capillary columns for LC/MS and SHORTFAST columns for rapid preparative separations.
- An enhanced website with comprehensive product descriptions and specifications including an extensive bibliography.
- Worldwide customer service from Grace's experienced international sales and support staff.

There's more to come in 2003: New products resulting from the union of Grace's expertise in silica science with The Separation Group's chromatography experience – providing innovative solutions for our customers' most demanding separations needs.

Folding

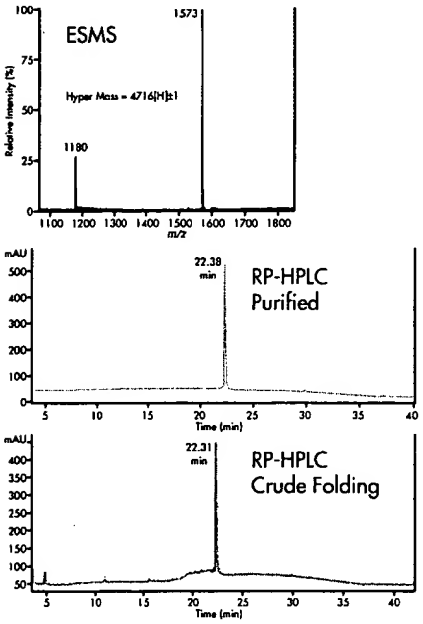

Figure 5. Reversed-phase and ESMS analysis of folded synthetic Crambin on VYDAC 214TP54 C4 column.

Data courtesy of:

Neeraj Chopra, Duhee Bang, and Stephen B.H. Kent
Dept. of Biochemistry and Molecular Biology
University of Chicago
Chicago, IL 60637 USA
Email: nchopra@delphi.bsd.uchicago.edu

Chromatographic Analysis of Insulin on VYDAC Small-Pore C8 Reversed-Phase Column

Analysis of recombinant insulin by reversed-phase HPLC was compared on three small-pore reversed-phase columns under identical conditions using a gradient of ACN in pH 4.25 ammonium acetate buffer for elution (Fig. 6). A VYDAC C8 column (208SP) provided excellent retention with shorter analysis times than the comparable VYDAC C18 column (201SP). A Kromasil C8 column operated under the same conditions provided retention for insulin comparable to the VYDAC C8, but produced slightly broader peaks leading to lower sensitivity (reduced peak height) and possibly lower resolution.

A 5x expansion of the absorbance scale for the C8 chromatograms (Fig. 7) reveals several small contaminating peaks. The VYDAC column is slightly more selective, and also demonstrates better resolution, particularly for contaminating peaks eluting immediately after the major insulin peak.

The VYDAC 208SP column had excellent stability under high-loading conditions. Even after 200 injections of insulin on the VYDAC C8 column (750 µg per injection), separations were very reproducible, with no observed loss of resolution nor any increase in backpressure (Fig. 8). Note that for this test the organic component of the mobile phase was *n*-propanol, an excellent solvent for large scale purifications.

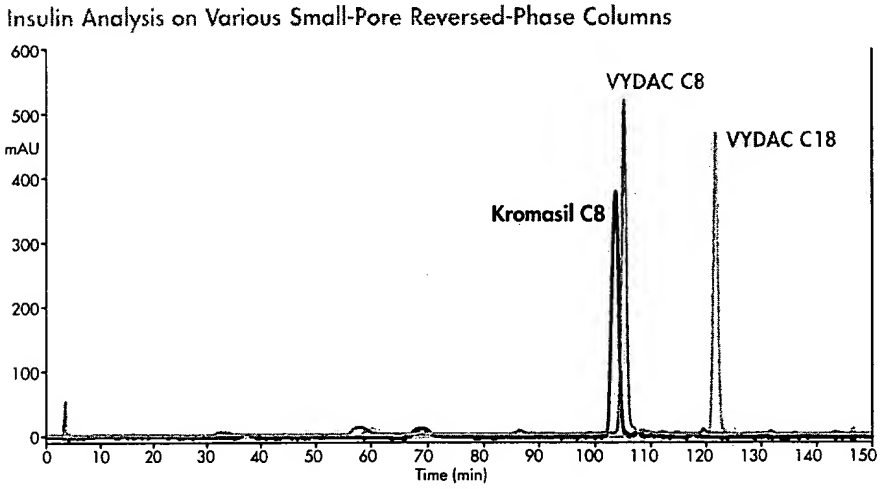

Figure 6. *Analytical separation of pharmaceutical grade insulin by reversed phase HPLC. Columns: VYDAC 208SP104 C8, VYDAC 201SP104 C18 (both 10 µm, 90 Å, 4.6 mm ID x 250 mm) and Kromasil C8 (10 µm, 100 Å, 4.6 mm ID x 250 mm). Identical conditions. Temperature: 29 °C. Flow rate: 0.8 mL/min. Mobile phase: A = 0.25 M NH_4OAc, pH 4.25. B = 40:60 solvent A:ACN. Gradient: 0 to 33% B in 10 minutes, then 33 to 53% B in 140 minutes. Injection: 50 µL containing 750 µg. Detection: UV, 276 nm.*

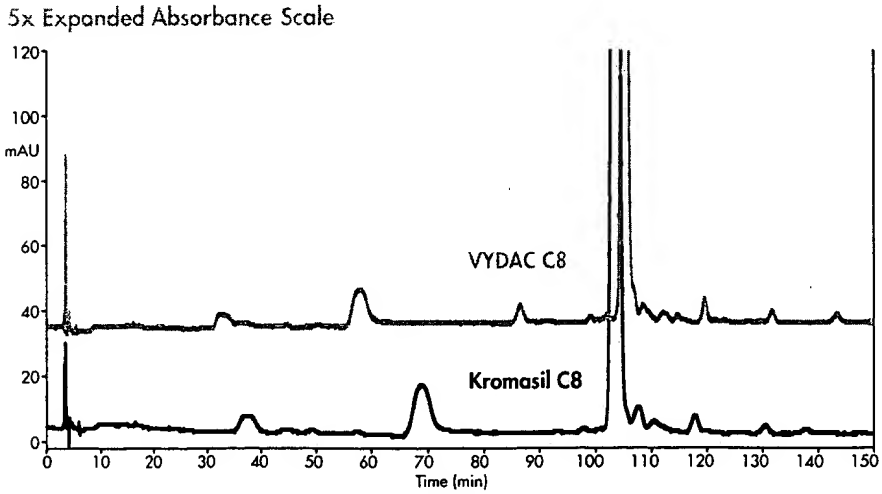

Figure 7. *C8 chromatograms of Figure 8 with absorbance scale expanded 5 fold.*

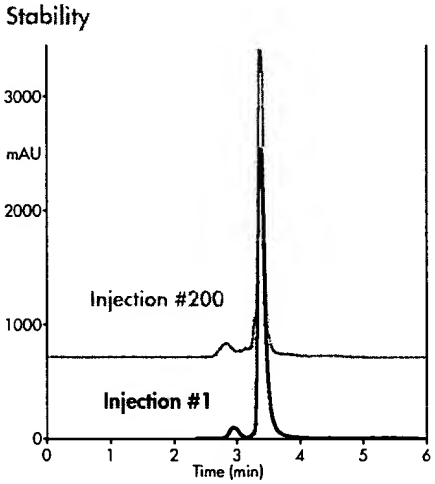

Figure 8. *Stability of insulin retention on VYDAC C8 column. Column: VYDAC 208SP104 (10 µm, 90 Å, 4.6 mm ID x 250 mm). Temperature: 25 °C. Flow rate: 1 mL/min. Mobile phase: A = 50 mM NH_4OAc, pH 4.2. B = 40:60 solvent A:n-propanol. Gradient: 35 to 42% B in 10 minutes. Maximum backpressure: 957 psi for injection #1 and #200. Sample: 50 µL containing 750 µg protein. Detection: UV, 276 nm.*

VYDAC® Polymer-Based Column Improves Recovery of Hydrophobic Synthetic Peptide

Efforts to produce by chemical synthesis desired quantities of the transmembrane segment of phospholamban (PLB), a membrane protein involved in regulation of calcium gradients across sarcoplasmic reticular membranes in cardiac muscle cells, have encountered difficulties in purification of the highly hydrophobic peptide. The PLB transmembrane peptide has the sequence: ARQNLQNLFINFCLILICLLLICIIVMLL. Its molecular mass is 3375.3. The amino acid composition is shown in Figure 9. The hydrophobic amino acids Ile and Leu comprise 20.7 and 31.0% of the residues, respectively.

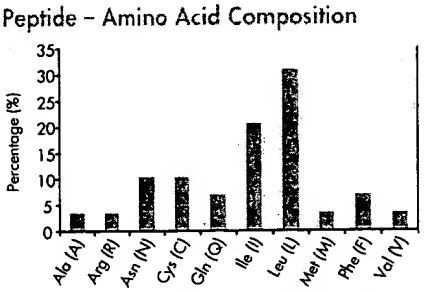

Figure 9. *Amino acid composition of PLB synthetic peptide.*

Poor recoveries of PLB synthetic peptide have been reported previously on silica-based reverse-phase columns. For example, the yield was 9% (based on starting resin) for a Phenomenex C4 column (Mayer et al., 1996). A yield of 12% (based on starting resin) was obtained from a VYDAC C18 (218TP54) column (Karim et al., 2000).

Recently, a research study at Miami University compared the purification of synthetic PLB transmembrane peptide on a VYDAC C4 silica-based reversed-phase with purification under identical conditions on a VYDAC 259VHP polystyrene-divinylbenzene polymer-based reversed-phase column (Fig. 10). Columns were equilibrated with 95% water, 2% acetonitrile and 3% 2-propanol. Peptide elution was achieved with a linear gradient to a final solvent composition of 5% water, 38% acetonitrile and 57% 2-propanol. The HPLC gradient that gave the best results for both columns was 5% - 95% B in 77 minutes.

The yield of pure peptide was 16% using the silica-based C4 column (based on 540 mg of peptide-resin). From the VYDAC polymer reversed-phase (259VHP5415) column the yield was 37% – more than twice the recovery from the any of the silica-based columns. MALDI-TOF mass spectrometry of the product purified by the 259VHP column confirmed its identification as the target peptide (Fig. 11). Recent larger scale purifications of this synthetic peptide on a VYDAC 259VHP82215 (8 µm, 300 Å, 22 mm ID x 150 mm) polymer-based preparative reversed-phase column have produced yields as high as 53%.

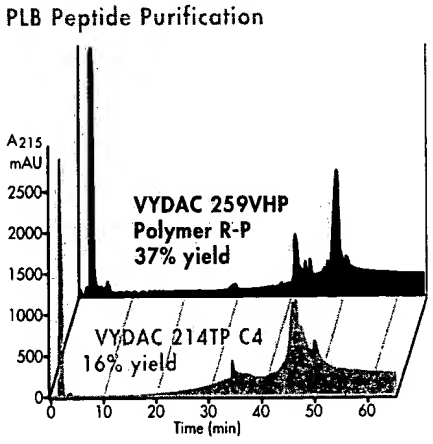

Figure 10. *HPLC separation of PLB synthetic peptide under identical conditions on a VYDAC 214TP104 (10 µm, 300 Å, 4.6 mm ID x 250 mm) C4 reversed-phase column and a VYDAC 259VHP5415 (5 µm, 300 Å, 4.6 mm ID x 150 mm) polymer reversed-phase column. (Conditions in text.)*

Advantages of VYDAC 259VHP polymer-based reversed-phase columns for purification of peptides with a high tendency to aggregate have been previously reported. (Refs. 3 and 4). They should always be considered as an alternative to silica-based reversed-phase columns wherever purification of difficult peptides is required.

References

1. Mayer, E. J., Mckenna, E., Garsky, V. M., Burke, C. J., Mack, H., Middaugh, C. R., Sardana, M., Smith, J. S., and Johnson, R. G., J. Biol. Chem. 271:1669-1677 (1996).
2. Karim, C. B., Marquardt, C. G., Stamm, J. D., Barany, G., and Thomas, D. D., Biochemistry, 39, 10892-10897 (2000).
3. Grace Vydac Application Note 9901, "Reversed-Phase Purification of Protein from a Recombinant Fusion."
4. Vydac Advances Newsletter, Fall 1999, page 5, "Vydac Reversed-Phase Columns Aid in Purification of Recombinant Alzheimer's Proteins."

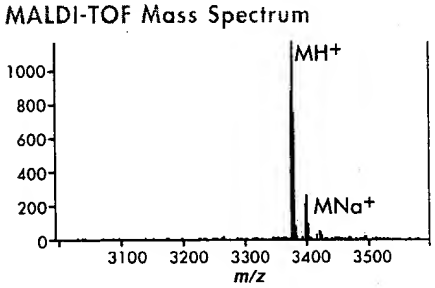

Figure 11. *MALDI-TOF mass spectrum of PLB synthetic peptide purified from the VYDAC 259VHP column. The peaks at m/z 3375 and at m/z 3398 represent the PLB peptide and its sodium adduct, respectively.*

Data courtesy of:

Elvis Tiburu and Gary A. Lorigan
Dept. of Chemistry and Biochemistry
Miami University
Oxford, Ohio 45056

GRACE VYDAC
17434 Mojave Street
Hesperia, CA 92345

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VYDAC Columns in This Issue

The VYDAC columns at right appear in applications described in this issue of *Grace Vydac Advances*. A listing of all available VYDAC HPLC columns can be found in the online store on the Grace Vydac website at www.gracevydac.com. Our online store has been updated for interactive searching by Catalog Number as well as a variety of other column characteristics. Each product listing includes complete description as well as links to application notes.

Orders for VYDAC products can be entered online, or by contacting your local VYDAC distributor or Grace Vydac World Headquarters:

Catalog No.	Description	Page No.
201SP104	Column, C18 Reversed Phase, 10 µm, 90 Å, 4.6 mm ID x 250 mm	4
208SP104	Column, C8 Reversed Phase, 10 µm, 90 Å, 4.6 mm ID x 250 mm	4
214TP54	Column, C4 Reversed Phase, 5 µm, 300 Å, 4.6 mm ID x 250 mm	2
214TP510	Column, C4 Reversed Phase, 5 µm, 300 Å, 10 mm ID x 250 mm	2
214TP104	Column, C4 Reversed Phase, 10 µm, 300 Å, 4.6 mm ID x 250 mm	5
218TP54	Column, C18 Reversed Phase, 5 µm, 300 Å, 4.6 mm ID x 250 mm	5
218TP104	Column, C18 Reversed Phase, 10 µm, 300 Å, 4.6 mm ID x 250 mm	1
259VHP5415	Column, Polymer Reversed Phase, 5 µm, 300 Å, 4.6 mm ID x 150 mm	5

Grace Vydac
17434 Mojave Street
Hesperia, CA 92345 USA

phone: (800) 247-0924 or (760) 244-6107
fax: (888) 244-6610 or (760) 244-1984
email: experts@vydac.com website: www.gracevydac.com